PATENT
Our Docket: P-ZA 3519

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Maurizio Zanetti	Group Art Unit: 1632
Serial No.: 09/300,959	Examiner: A. Beckerleg
Filed: April 27, 1999	
For: SOMATIC TRANSGENE IMMUNIZATION AND RELATED METHODS	

Commissioner for Patents
Washington, D.C. 20231

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Sir:

I, the undersigned, declare as follows:

1) I am the Maurizio Zanetti who is named as the inventor on the above-identified patent application.

2) I understand that the claims stand rejected, in part, as allegedly lacking enablement for treating a variety of diseases and conditions.

Administration of nucleic acids Intrasplenically induces protective immunity against influenza A virus infection

3) The class I-restricted peptide $NP_{366-374}$ (ASNENMETM) of influenza A virus is immunodominant and induces protective immunity in mice. Plasmid $\gamma 1NP^3$ was created by inserting the $NP_{366-374}$ polypeptide into the third complementarity-determining region (CDR) of the murine IgH heavy chain gene V_H62 and cloning

EXHIBIT B

the resulting V region into the expression vector pSVneoγ1 coding for the human IgG1 constant region under the control of the IgH promoter and enhancer elements. Plasmid $\gamma 1NV^2NP^3$ is identical to $\gamma 1NP^3$ except that it also contains the heterologous Th cell determinant, NANPNVDPNANP inserted in CDR2. Mice were immunized with 100 µg of plasmid in PBS by single intra-spleen inoculation.

4) As shown in Exhibit 1, mice immunized *in vivo* with plasmid $\gamma 1NP^3$ or plasmid $\gamma 1NV^2NP^3$ developed cytotoxic T lymphocytes (CTL) specific for $NP_{366-374}$. Twenty one days after immunization with plasmid $\gamma 1NP^3$ (Group II), 66% of mice developed CTL specific for $NP_{366-374}$, comparably better than the levels induced at the same time for mice immunized with synthetic $NP_{366-374}$ peptide (Group I). Further improvement in CTL induction was observed 21 days after immunization with plasmid $\gamma 1NV^2NP^3$ (Group III), with 93% of mice developing CTL specific for $NP_{366-374}$. Moreover, as shown in the last column of Exhibit 1, responders immunized with the $\gamma 1NV^2NP^3$ had, on average, a two fold higher percent specific lysis than responders immunized with synthetic $NP_{366-374}$ peptide.

5) As shown in Exhibit 2, *in vivo* immunization with $\gamma 1NP^3$ provided protection against acute infection from influenza virus. Mice (10 per group) were immunized either with a single intraspleen inoculation of $\gamma 1NP^3$ (closed diamonds), intraspleen inoculation of $\gamma 1NP^3$ followed by subcutaneous immunization with

synthetic peptide $NP_{366-374}$ (closed triangles), or two subcutaneous immunizations of synthetic peptide $NP_{366-374}$ (X). Untreated mice (open squares) served as a control. All mice were infected by intranasal inoculation with a $10xLD_{50}$ infectious dose of A/PR8/34 (MA) influenza virus 180 days after priming. All non-vaccinated mice died by day 7, all mice immunized with control plasmid pSVneoγ1 died by day 11, and all peptide-immunized mice died by day 11. In contrast, 60% of mice immunized with plasmid $\gamma1NP^3$ followed by $NP_{366-374}$ booster and 50% of mice immunized with plasmid $\gamma1NP^3$ alone resisted virus challenge for the 30 day observation period.

6) As shown in Exhibit 3, *in vivo* immunization with $\gamma1NV^2NP^3$ protected mice against acute infection when challenged with very high doses of influenza virus. Mice (7-10 per group) were immunized either by intraspleen inoculation of $\gamma1NP^3$ (closed diamonds), $\gamma1NV^2NP^3$ (closed squares), $\gamma1NP^3\Delta L11$ (closed triangles), or with two subcutaneous immunizations of synthetic peptide $NP_{366-374}$ (X). Untreated mice (open squares) served as a control. All mice were infected with intranasal inoculation of a $20xLD_{50}$ infectious dose of A/PR8/34 (MA) influenza virus 80 days after priming. All non-vaccinated and all peptide-immunized mice died within 9 days of challenge. Mice vaccinated with the leader sequence deletion $\gamma1NP^3\Delta L11$ plasmid also died by day 9. In contrast, vaccination using the dual epitope $\gamma1NV^2NP^3$ plasmid afforded protection in 60% of mice after 28 days and $\gamma1NP^3$ plasmid-vaccinated mice were marginally protected.

Administration of ex vivo transfected cells induces protective immunity against influenza A virus infection

7) CTL responses were observed in mice immunized with transgenic $\gamma 1NV^2NP^3$ spleen lymphocytes. C57B1/6 spleen lymphocytes were transfected *ex vivo* with plasmid $\gamma 1NV^2NP^3$, and $1x10^3$ to $2x10^4$ of the transgenic spleen cells were intravenously administered to C57B1/6 mice. In three independent experiments, a specific and in most instances strong CTL response was measured in 39 out of 39 (100%) mice starting from day 14.

8) As shown in Exhibit 4, *ex vivo* immunization with transgenic $\gamma 1NV^2NP^3$ spleen lymphocytes provided complete protection against acute lethal infection from influenza virus. C57B1/6 mice were intravenously immunized with transgenic spleen lymphocytes and challenged after twenty one days with a lethal intranasal dose of influenza virus. All control mice inoculated with syngeneic lymphocytes transgenic for plasmid pSVneoγ1 (open squares) died by day 9. In contrast, all mice vaccinated with lymphocytes transgenic for $\gamma 1NV^2NP^3$ resisted the challenge and survived for the full 40 day observation period.

9) As shown in Exhibit 5, administration of as few as 300 lymphocytes transgenic for $\gamma 1NV^2NP^3$ protected mice from lethal influenza A virus infection. In contrast, mice vaccinated with lymphocytes transgenic for control plasmid $\gamma 1NV^2NA^3$, identical to $\gamma 1NV^2NP^3$ with the exception of containing the antigen of

Plasmodium falciparum in place of the Influenza Virus $NP_{366-374}$ peptide, were not protected from lethal influenza A infection.

Administration of nucleic acids Intrasplenically induces protective immunity against experimental allergic encephalomyelitis

10) Experimental allergic encephalomyelitis is a rodent model of multiple sclerosis. The T cell receptor (TCR) of autoreactive T lymphocytes, specific for myelin basic protein, mediates the pathogenesis of the disease. Plasmids expressing a TCR vaccine were prepared as follows. The γ1TCRI plasmid was produced by inserting the rat Vb8.2 CDR3 peptide (ASSDSSNTE) into CDR3 of an IgH heavy chain gene and the γ1TCRII plasmid was produced by inserting the rat Vb8.2 CDR2 peptide (DMGHGLRLIHYSYDVNSTEKG) into CDR3 of an IgH heavy chain gene. A schematic representation of the modified H chains is shown in Exhibit 6. Plasmid construction and intra-spleen inoculation were performed using methods similar to those described in paragraph 3.

11) As shown in Exhibit 7, the incidence of experimental allergic encephalomyelitis was prevented in rats inoculated with γ1TCRII. Furthermore, the severity of disease (disease index) was markedly reduced for rats inoculated with γ1TCRII plasmid. Similarly, rats given both the γ1TCRI and γ1TCRII plasmids were also protected.

12) Exhibit 8 shows the distribution of experimental allergic encephalomyelitis according to severity score. Only 45% of rats receiving γ1TCRII (Groups II and III) developed severe experimental allergic encephalomyelitis (score ≥3), significantly fewer than the 87% of non-inoculated control rats (Group VI) or 83% of rats inoculated with the γ1WT control plasmid (Group V). Furthermore, none of the rats receiving γ1TCRII had a severity greater than 3.

13) Exhibit 9 shows that the average duration of severe disease, in γ1TCRII-inoculated rats, was shorter than in any other group. Notably, only one rat developed experimental allergic encephalomyelitis with a severity score of 3 lasting more than 2 days. In control rats, 13 of 16 (81%) had a duration of experimental allergic encephalomyelitis in grade 3 for 3 days or longer (data not shown).

Administration of ex vivo transfected cells induces protective immunity against B16-MUC.1 tumors

14) Mucin 1 is a glycoprotein expressed in tumors of epithelial origin in humans. C57B1/6 mice are non-tolerant of mucin 1, while MUC.1 transgenic mice, expressing the glycoprotein, are mucin 1 self tolerant. Immunization of mice with mucin 1 was carried out with plasmid $\gamma 1NV^2VTSA^3$, which expresses an IgH heavy chain containing a MUC-1 epitope in CDR3 and a Th cell determinant, NANPNVDPNANP in CDR2 under the control of IgH promoter and enhancer elements. Plasmid $\gamma 1NV^2NA^3$ is

identical to $\gamma 1NV^2VTSA^3$ with the exception of containing the antigen of *Plasmodium falciparum* in place of the MUC-1 epitope. C57B1/6 spleen lymphocytes were transfected *ex vivo* and administered to mice using methods similar to those described in paragraph 7.

15) As shown in Exhibit 10, CD4 T cell proliferation was induced in both C57B1/6 (non-tolerant) mice and MUC.1 transgenic (tolerant) mice immunized with transgenic $\gamma 1NV^2VTSA^3$ lymphocytes. The response was against both the dominant and sub-immunogenic MUC-1 determinant and was comparable to the response induced in non-tolerant C57B1/6 mice. For spleen lymphocytes harvested two weeks after immunization, the responses were significantly higher than observed for control immunizations with γ^1TL540 lymphocytes expressing a determinant of human telomerase reverse transcriptase.

16) As shown in Exhibit 11, mice immunized with transgenic $\gamma 1NV^2VTSA^3$ lymphocytes were protected from tumor formation. MUC.1 transgenic mice were immunized with lymphocytes transgenic for either $\gamma 1NV^2VTSA^3$ (closed squares), $\gamma 1NV^2NA^3$ (closed triangles) or pSVneo (open squares), receiving a first immunization of $5x10^3$ transgenic lymphocytes on day 0 followed by a similar immunization on day 21. Mice were challenged three days after the second immunization with $2x10^4$ B16-MUC.1 melanoma tumor cells or B16 neo melanoma tumor cells subcutaneously. Control mice immunized with non transgenic lymphocytes (open triangles) were also challenged. The number of mice in each group is indicated in parenthesis in Exhibit 11. All 16 mice

vaccinated with transgenic $\gamma 1NV^2VTSA^3$ lymphocytes and challenged with B16-MUC.1 tumor cells were protected, surviving through the 8 month observation period tumor-free. In contrast, all mice vaccinated with control transgenic lymphocytes developed tumors and expired before 30 days. Mice vaccinated with lymphocytes transgenic for $\gamma 1NV^2VTSA^3$ and challenged with the B16 neo tumor cells lacking the mucin 1 antigen (asterisks) also formed tumors and died, indicating that the protection against B16-MUC.1 tumor challenge was specific for the mucin 1 antigen.

17) As shown in Exhibit 12, a subsequent challenge of B16-MUC.1 protected mice with tumor cells demonstrated that protection was long lived and specific. To this end, 70 days after the first challenge with B16-MUC.1 tumor cells, 12 protected mice (immunized with transgenic $\gamma 1NV^2VTSA^3$ lymphocytes) were divided into two groups and challenged again with either B16-MUC.1 (closed squares) or B16 neo (open squares) tumor cells. Mice re-challenged with the MUC.1-expressing tumor continued to be protected. In contrast, mice that received B16 neo tumor cells died like controls that had been immunized with nontransgenic lymphocytes (open triangles).

18) In summary, treatment of a variety of pathological conditions using the methods of the invention has been demonstrated. In addition, to those exemplified in the specification, the results described above show stimulation of an immune response for treatment of influenza A virus infection, encephalomyelitis and cancer. The results described above also

demonstrate successful *in vivo* and *ex vivo* administration of nucleic acids.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Date: 11/08/02 By: _____

Maurizio Zanetti

EXHIBIT B